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The *in Vivo* Role of Androgen Receptor SUMOylation as Revealed by Androgen Insensitivity Syndrome and Prostate Cancer Mutations Targeting the Proline/Glycine Residues of Synergy Control Motifs*

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Background: Androgen receptor (AR) SUMOylation inhibits transactivation, but the impact on human disease is unknown.
Results: AR mutations associated with androgen insensitivity syndrome and prostate cancer affect AR SUMOylation and function.
Conclusion: Altered SUMOylation is the molecular basis of specific AR-based human diseases.
Significance: AR SUMOylation is an important physiological mechanism *in vivo* that could be targeted for therapy.

The androgen receptor (AR) mediates the effects of male sexual hormones on development and physiology. Alterations in AR function are central to reproductive disorders, prostate cancer, and Kennedy disease. AR activity is influenced by post-translational modifications, but their role in AR-based diseases is poorly understood. Conjugation by small ubiquitin-like modifier (SUMO) proteins at two synergy control (SC) motifs in AR exerts a promoter context-dependent inhibitory role. SC motifs are composed of a four-amino acid core that is often preceded and/or followed by nearby proline or glycine residues. The function of these flanking residues, however, has not been examined directly. Remarkably, several AR mutations associated with oligospermia and androgen insensitivity syndrome map to Pro-390, the conserved proline downstream of the first SC motif in AR. Similarly, mutations at Gly-524, downstream of the second SC motif, were recovered in recurrent prostate cancer samples. We now provide evidence that these clinically isolated substitutions lead to a partial loss of SC motif function and AR SUMOylation that affects multiple endogenous genes. Consistent with a structural role as terminators of secondary structure elements, substitution of Pro-390 by Gly fully supports both SC motif function and SUMOylation. As predicted from the functional properties of SC motifs, the clinically isolated mutations preferentially enhance transcription driven by genomic regions harboring multiple AR binding sites. The data support the view that alterations in AR SUMOylation play significant roles in AR-based diseases and offer novel SUMO-based therapeutic opportunities.

The physiological and pathophysiological effects of androgens are mediated by the androgen receptor (AR),[3] a member of the nuclear hormone receptor superfamily of transcription factors. AR is essential for normal primary male sexual development during prenatal and early postnatal phases and for expression and maintenance of secondary sexual characteristics upon reaching puberty. AR dysfunction in XY individuals is thus associated with multiple human diseases. Nearly 900 clinically isolated AR mutations have been identified (1), and the majority (~80%) are associated with androgen insensitivity syndrome (AIS), a spectrum of disorders of male sexual differentiation and function. Depending on the location and severity of the mutation, AIS can range from mild defects in spermatogenesis in otherwise normal males to individuals with a completely female appearance (2, 3). Although most clinically isolated AR mutations map to the central DNA binding and C-terminal ligand binding domains of the receptor, the majority of the mutations in exon 1 or the N-terminal region of the receptor are associated with AIS (1).

AR dysfunction is also associated with prostate cancer, the second most common cancer in men (4). In prostate cancer cells, which have accumulated initiating and promoting genetic alterations, androgens provide the major survival and proliferative drive that sustain the progression of the disease (5). Despite an initial favorable response to androgen deprivation or antiandrogen therapy, tumors nearly invariably relapse within 18–36 months (6) as castration-resistant (also termed androgen-independent or hormone-refractory) prostate cancer. Notably, in most cases, proliferation at this stage remains dependent on the presence and activity of AR, which persists through multiple mechanisms, including AR gene amplification, broadening of AR ligand specificity, or through post-translational alterations that directly activate or sensitize the

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[3] The abbreviations used are: AR, androgen receptor; SUMO, small ubiquitin-like modifier; SC, synergy control; AIS, androgen insensitivity syndrome; ARE, androgen-response element.

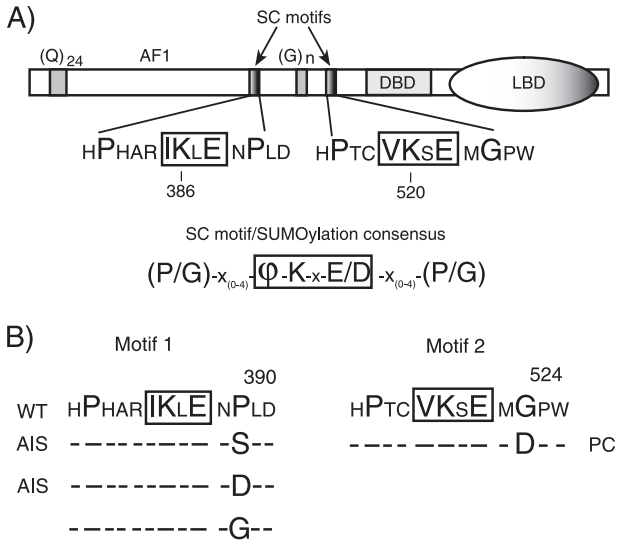

FIGURE 1. **AR harbors two conserved synergy control motifs.** *A,* schematic representation of human AR highlighting key structural features and the two SC motifs and the two SUMOylation sites. The core SC motifs are *boxed* with the SUMO acceptor lysines (Lys-386 and Lys-520) indicated. Flanking Pro/Gly residues are in *larger type*. Numbering is based on Ref. 57. The modified lysines are often preceded and/or followed by nearby proline or glycine residues. The proline and the glycine residues downstream from the first and the second SC motif are *highlighted*. The SC motif/SUMOylation consensus sequence is shown below. *B,* clinical mutations associated with AIS and prostate cancer (*PC*) map to Pro-390 and Gly-524, respectively.

receptor (7). Multiple AR mutations have been recovered from prostate cancer samples, and although most studies have focused on the ligand binding domain, a significant number of mutations have also been identified in the N-terminal region (8–11). Although the prevalence of such mutations and their role on disease progression have been unclear, recent data clearly indicate that treatment with antiandrogens specifically selects for gain-of-function AR mutations with altered stability, promoter preference, or ligand specificity (12).

Although androgen binding is considered the main mechanism for controlling the activity of the androgen receptor, post-translational mechanisms exert significant effects on its activity. Our group initially identified a regulatory amino acid motif (SC motif) found in multiple transcription factors that exerts a promoter context-dependent inhibitory effect and restrains the transcriptional activation of factors when stably bound to multiple, closely spaced instances of their cognate response element. In contrast, SC motifs are functionally silent when activators are bound to a single site (13–15). Two copies of this motif are present in the N-terminal region of AR (Fig. 1*A*) (15). Subsequently, we (14, 16) and others (17) demonstrated that these motifs function by serving as sites for post-translational modification by small ubiquitin-like modifier (SUMO) proteins. Mutation of the acceptor lysines within the motifs in AR (K386R/K520R) prevents SUMO conjugation and relieves inhibition, leading to a significant enhancement of AR activity.

SUMO proteins (SUMO1–3) are structurally related to ubiquitin (18, 19) and are reversibly conjugated to target proteins through an enzymatic pathway analogous to ubiquitination but carried out by a distinct SUMO-specific set of enzymes. The transfer of SUMO to the target protein is performed by the SUMO-specific E2-conjugating enzyme Ubc9 (20), and this step is facilitated by a growing number of SUMO E3 ligases such as RanBP2 and members of the protein inhibitor of activated STAT (PIAS) family (21–23). SUMO conjugation is reversed through the isopeptidase activity of a unique set of SUMO-specific proteases also known as SENPs (24). Studies by our group and others have shown that SUMO inhibits the activity of transcription factors because it harbors an intrinsic repressive function (14). Through an extensive mutagenesis, we mapped this function to a distinct conserved pocket in the surface of SUMO (25). This pocket serves as a binding site for short SUMO-interacting motifs in partner proteins (26). Multiple transcription cofactors as well as chromatin modification and remodeling components bearing such SUMO-interacting motifs have been implicated in SUMO-mediated inhibition of transcription (27).

SC motifs consist of a four-amino acid SUMOylation core that includes the modified lysine and is usually preceded and/or followed by nearby proline or glycine residues (13, 15). The *in vivo* functional impact of the core and flanking SC motif residues in AR and whether SC motif function is associated with AR-based diseases have not been examined. Remarkably, a nonsynonymous cytidine to thymidine substitution in exon 1 of *AR* that leads to a missense P390S mutation has been isolated repeatedly in independent patients with partial AIS. The mutation was described in two unrelated patients with oligospermia (3) and subsequently in an infertile male (28). Very recently, the mutation was isolated in a pediatric patient with hypospadias (29) and in a patient with micropenis (30). This exact mutation was also isolated in a patient with testicular cancer (31). Similarly, a P390D (32) and a P390R in combination with Q443R (8) were reported to be associated with complete AIS. Pro-390 lies immediately downstream of the core of the first SC motif (Fig. 1*B*). Similarly, a mutation (G524D) in the equivalent position of the second motif was isolated from a tumor sample of a patient with castration-resistant prostate cancer (Gleason score 7) (Fig. 1*B*) (10). These findings, coupled with our recent demonstration that AR SUMOylation can prevent hormone-dependent aggregation of polyglutamine-expanded androgen receptor, a critical step in Kennedy disease pathogenesis (33), prompted us to examine the mechanistic effects of clinically isolated Pro/Gly mutations in SC motifs especially because they provide a way to probe the *in vivo* impact of AR SUMOylation in the context of the AR-based human diseases AIS and prostate cancer.

EXPERIMENTAL PROCEDURES

Mammalian Expression and Reporter Plasmids—Cytomegalovirus promoter-driven expression vectors for AR mutants are derivatives of plasmid p5HB human AR encoding WT human AR bearing a 24-glutamine tract (33). Mutations in each SC motif were introduced using the QuikChange site-directed mutagenesis approach. Combinations of mutations in both SC motifs were created by swapping KpnI/HindIII or RsrII/HindIII fragments between constructs. Sequences for WT AR and mutants were also subcloned into the pCDNA5 FRT vector (Invitrogen). All manipulated regions were confirmed by sequencing. The pCMV-driven (pcDNA3) expression vector for HA-tagged SUMO3 has been described previously (14). The pΔODLO reporter plasmid in which a minimal *Drosophila* dis-

tal alcohol dehydrogenase promoter (−33 to +55) drives the firefly luciferase gene, as well as its derivatives, pΔ$(TAT)_1$-Luc and pΔ$(TAT)_4$-Luc, that harbor one or four copies of a minimal androgen-response element (ARE) from the tyrosine aminotransferase (*TAT*) gene have been described previously (15). pRSV β-gal is a Rous sarcoma virus promoter-β-galactosidase expression vector and was used to correct for transfection efficiency. The panel of luciferase-based reporters harboring 500-bp genomic regions centered around genomic sites occupied by AR *in vivo* has been described previously (34, 35).

Cell Culture, Transfections, and in Vivo SUMOylation—Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 5% charcoal-stripped fetal bovine serum. Stable cell lines expressing both wild type and mutant forms of human AR were generated using the FlpIn system (Invitrogen). In brief, 293FlpIn cells (1.2×10^5/well) were seeded onto 24-well plates and co-transfected with 720 ng of the Flp recombinase expression vector, pOG44, and 80 ng of one of the pCDNA5 FRT human AR constructs using Lipofectamine 2000. Cells were cultured for at least a week in 150 μg/ml hygromycin in growth medium to select stable transfectants. For functional assays, 293T cells were seeded onto 96-well plates (5×10^3/well) and transfected 24 h later using Lipofectamine (Invitrogen). For experiments using the TAT-derived reporters, transfections included 1 ng of the indicated AR expression plasmid, 30 ng of reporter plasmid, and 1 ng of the control pRSV β-gal plasmid. For functional assays using the panel of luciferase reporters harboring 500-bp AR binding regions, transfections included 0.1 ng of AR expression plasmid and 30 ng of reporter plasmid. For certain reporters that displayed significantly elevated basal and/or AR-stimulated activity, the amount of AR expression or reporter plasmid was reduced as indicated in the figure legend. In all cases, the total amount of DNA was supplemented to 90 ng/well with pBSKS(−) and the empty vector pCMV-5 so as to maintain equimolar amounts of each type of expression plasmid. After 16 h, cells were treated with 10 nM R1881 or vehicle (0.1% ethanol). Cells were lysed 20 h later, and luciferase and β-galactosidase activities were determined as described (36). To assess AR expression levels, cells in parallel wells were transfected as for the functional assays, lysed in 4× SDS-PAGE sample buffer, resolved by 7.5% SDS-PAGE, and processed for immunoblotting as described below. For endogenous gene expression analysis, stable AR expressing lines were seeded onto 96-well plates (20,000 cells/well) and incubated for 16 h prior to treatment with either vehicle or 50 nM R1881 for an additional 6 h. Cells were subsequently harvested for RNA isolation using RNeasy kits (Qiagen). cDNA was synthesized using iScript (Bio-Rad). Quantitative real time PCRs were carried out in duplicate in a 480 LightCycler (Roche Diagnostics) using QuantiTect SYBR Green reagents (Qiagen) and primers for human *RPL19* (forward 5′-ATGTATCACAGCCTGTACCTG-3′ and reverse 5′-TTCTTGGTCTCTTCCTCCTTG-3′); *S100P* (forward 5′-CGGAACTAGAGACAGCCATGGGCAT-3′ and reverse 5′-AGACGTGATTGCAGCCACGAACAC-3′); *TMEM37* (forward 5′-CGCCGGGCGCAGCATGA-3′ and reverse 5′-CCACAGCCAGGGCCACACA-3′); *ACSL1* (forward 5′-ACAAGTGGAACTACAGGCAACCCCA-3′ and reverse 5′-CCAAGAGCCATCGCTTCAGCGT-3′); *CREB3L2* (forward 5′-TGACCATCACAGCCATCTCCACCC-3′ and reverse 5′-ACTCAGGCTGCCCTCTGAGTCACTG-3′); *FKBP5* (forward 5′-GGAATGGTGAGGAAACGCCG-3′ and reverse 5′-CTCTCCTTTCTTCATGGTAGCCAC-3′); and *IGFBP1* (forward 5′-TGATGGCCCCTTCTGAAGAG-3′ and reverse 5′-CCTTCGAGCCATCATAGGTACTG-3′) genes. LinRegPCR (Version 11.0) software was used to estimate the mRNA levels of the target genes relative to those of RPL19. For *in vivo* SUMOylation experiments, HEK293T cells were seeded in 6-well plates (1.5×10^5/well) and transfected with 0.5 μg of the indicated AR expression plasmids and 0.5 μg of pCDNA3 HA-SUMO-3 using FuGENE-6® transfection reagent. Cultures were supplemented with 10 nM R1881 or vehicle (0.1% ethanol) 24 h post-transfection and harvested 20 h later. After processing and AR immunoprecipitation (33), samples were resolved by 7.5% SDS-PAGE and processed for immunoblotting as described below.

In Vitro SUMO Conjugation and De-conjugation Assays—Immunopurified AR forms for use as SUMOylation substrates were generated as follows. 293T cells in 10-cm plates (1×10^6 cells/plate) were transfected using the calcium phosphate method with 5 μg of the indicated AR constructs and 5 μg of pBSKS(−). Cells were exchanged to fresh medium after 12 h and exposed to 10 nM R1881 for an additional 4 h. Cells were lysed for 15 min on ice with 1 ml of high salt lysis buffer (20 mM Hepes (pH 7.5), 400 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1% Nonidet P-40) containing 1 tablet per 10 ml of Complete™ protease inhibitors (Roche Diagnostics). Extracts were then supplemented with 20 mM *N*-ethylmaleimide and further incubated 5 min on ice. After addition of 8 μl of rabbit polyclonal AR-N20 antibody, immune complexes were recovered with 100 μl of a 50% suspension of protein A-agarose (Invitrogen) at 4 °C for 2 h. Immunoprecipitates were washed three times in low salt lysis buffer (200 mM NaCl) and resuspended to a final volume of 100 μl (50% slurry). SUMOylation reactions were carried out in 30 μl of 50 mM Tris (pH 7.5), 5 mM $MgCl_2$ and in the presence of 0.25 μM purified His-tagged SUMO E1, 1 μM GST-Ubc9, 10 μM His-SUMO-1, and 12 μl of immunopurified AR (50% slurry). Reactions were initiated by the addition of an ATP regeneration system (final concentrations: 10 units/ml creatine kinase, 25 mM phosphocreatine, 5 mM ATP, and 0.6 units/ml pyrophosphatase). Reactions were incubated at 37 °C with agitation for the indicated times, terminated by addition of 5 μl of 4× disruption buffer (50 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 0.24 M β-mercaptoethanol, 0.015% bromphenol blue), and boiled for 5 min. Samples were resolved by 7.5% SDS-PAGE and processed for immunoblotting as described below. For deconjugation experiments, immunopurified SUMOylated AR forms were obtained as described above for the conjugation assay except that cells were transfected with 5 μg of pCDNA3 HA-SUMO3 in place of pBSKS(−). To preserve SUMOylation, cells were lysed in the presence of 20 mM *N*-ethylmaleimide for 15 min on ice. *N*-Ethylmaleimide was then quenched by addition of dithiothreitol to 40 mM final concentration and a further 2-min incubation on ice. After immunoprecipitation and washing, beads were resuspended (50% slurry) in reaction buffer (25

mM Tris HCl (pH 8.0), 150 mM NaCl, 0.1% Tween 20, and 2 mM DTT). Deconjugation reactions (30 μl) were carried out at 25 °C and included 15 μl of AR beads (50% slurry) and 26 nM of purified WT or catalytically inactive (C548S) SENP2. Reactions were terminated and samples processed as for the conjugation reaction.

Immunoblotting and Quantitation—Following SDS-PAGE, samples were transferred to Immobilon-P (Millipore). Membranes were probed with primary rabbit polyclonal AR-N20 (Santa Cruz Biotechnology) or mouse monoclonal HA-11 (Covance) antibodies followed by goat anti-rabbit or anti-mouse IgG peroxidase-conjugated (Bio-Rad) secondary antibodies. Immunoreactive proteins were detected by chemiluminescence using SuperSignal West Femto substrates (Pierce), and images were captured in a Kodak Image Station 440 CF. For quantitation of AR SUMOylation, the anti-AR signal derived from the SUMO-modified forms was normalized to the total AR signal (unmodified + SUMO modified). For Figs. 3 and 4, values obtained for individual mutants are expressed as a percentage of the SUMOylation observed for WT AR.

Expression and Purification of Recombinant Proteins—BL21 DE3-CodonPlus cells harboring the pGEX-hUbc9 expression vector and BLR (DE3) pLysS cells (Novagen) harboring plasmids pET15bHis SUMO-1, pDuet (Amp) His-huAos1/Uba2, pET28b His6-SENP2, or pET28b His6-SENP2 C548S were grown at 37 °C in LB medium containing 25 μg/ml chloramphenicol, 12 μg/ml tetracycline, and 50 μg/ml of either carbenicillin (for SUMO1) or kanamycin (for SENP2 constructs). Cultures in logarithmic growth phase were induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside for 2 h at 37 °C. Cells were centrifuged at 8,000 × *g* for 15 min at 4 °C. For GST Ubc9, the pellet was resuspended in buffer A (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol, 10% glycerol, and Complete Mini™ protease inhibitor mixture tablets (1 tablet/10 ml)). After lysozyme treatment (40 μg/ml for 60 min on ice) and sonication at 4 °C, the lysate was centrifuged at 35,000 rpm at 4 °C for 30 min. The supernatant was incubated with 2 ml of glutathione-agarose (Sigma) for 60 min at 24 °C. The resin was washed with 10 bed volumes of buffer A without protease inhibitors and with 10 bed volumes of buffer B (buffer A with 400 mM NaCl). Proteins were eluted in buffer B supplemented with 20 mM reduced glutathione. For His-tagged SUMO-1, SENP2 WT and mutant, and Aos1/Uba2, cells were processed as above in buffer C (50 mM sodium phosphate buffer (pH 8.0), 300 mM NaCl, 10% glycerol, 10 mM imidazole, 5 mM β-mercaptoethanol, EDTA-free Complete Mini™ protease inhibitor mixture tablets (1 tablet/10 ml)). Extracts were incubated with 2 ml of nickel-nitrilotriacetic acid resin (Qiagen) for 1 h at 4 °C. The resin was washed with 10 bed volumes of buffer C, followed by 2 bed volumes of buffer C containing 20 mM imidazole. Proteins were eluted in buffer C containing 250 mM imidazole. All proteins were exchanged into buffer D (10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 5 mM dithiothreitol, 20% glycerol) by gel filtration and stored at −80 °C until use.

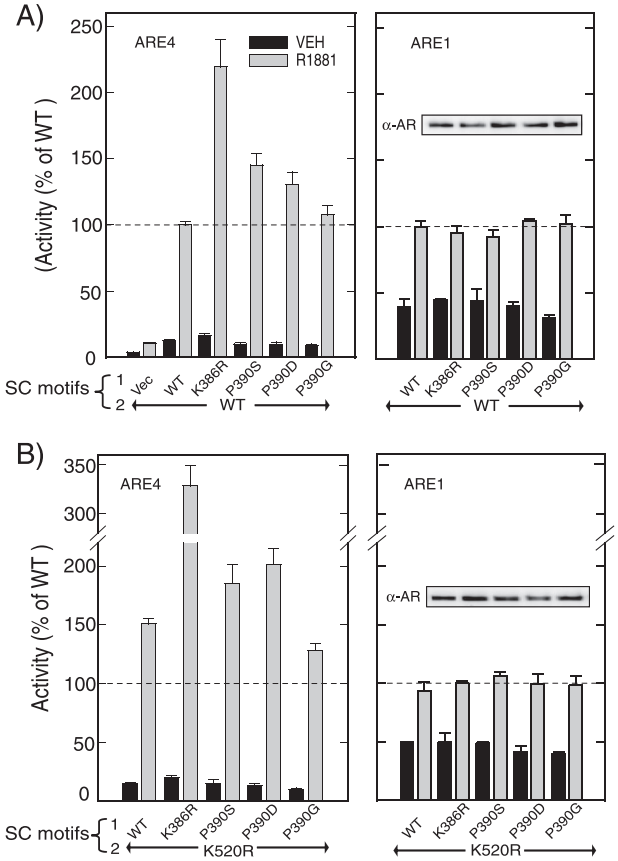

FIGURE 2. **Mutations of Pro-390 in AR that are associated with AIS lead to partial loss of SC motif function.** 293T cells were co-transfected as indicated under "Experimental Procedures" with expression vectors for the indicated AR mutants and the control pRSV β-gal expression vector (1 ng each) together with 30 ng of pΔ(TAT)$_4$-Luc (ARE$_4$, *left panel*) and pΔ(TAT)$_1$-Luc (ARE$_1$, *right panel*) reporter plasmids, respectively. Cells were treated with vehicle or 10 nM of the AR agonist R1881 and assayed as described under "Experimental Procedures." *A,* transcriptional activity of AR bearing the indicated mutations in the first SC motif at ARE$_4$ (*left panel*) and ARE$_1$ (*right panel*). The P39S and P39D mutations are associated with AIS. *B,* effect of the same mutations as in *A,* but in the context of a disabled second SC motif due to mutation of the SUMO acceptor lysine (K520R) is shown. Data represent the means ± S.E. of at least four to five independent experiments performed in triplicate and are expressed as a percentage of the WT activity (28.7 ± 4.6 for ARE$_4$ and 4.66 ± 0.26 for ARE$_1$). Western blot analysis of the expression of WT and mutant AR forms is shown in the *insets*.

RESULTS

Pro-390 Mutations Associated with AIS Affect Synergy Control Function and SUMOylation—The inhibitory effect of SC motifs is promoter context-dependent because disruption of the motifs enhances AR activity at promoters bearing multiple AR response elements but has no effect from those bearing a single site (15, 33). To assess whether the clinically isolated Pro-390 mutations affect the function of the first SC motif, we examined the effect of the mutations at two promoters differing only on the number of AR-binding sites. As can be seen in Fig. 2*A*, and consistent with previous data, substitution of the SUMO acceptor Lys-386 by arginine enhanced AR transcriptional activity by nearly 2.5-fold compared with the WT receptor at the promoter bearing four closely spaced AR-binding sites (ARE$_4$). In contrast, no effect was observed at a promoter bearing an isolated AR-binding site (ARE$_1$). Notably, the P390S and P390D mutations associated with oligospermia and com-

plete AIS, respectively, also displayed a selective enhancement at the ARE4 promoter. Consistent with a partial loss of SC motif function, the magnitude of the effect was less pronounced than the K386R mutation. The definition of SC motifs allows for either proline or glycine residues at this position. Therefore, we also examined the effect of substituting Pro-390 by glycine. Consistent with the description of SC motifs, this mutation had no discernible effect on either promoter. Given that AR harbors two SC motifs, we examined the effect of the same mutations in the context of a disabled second motif. As can be seen in Fig. 2*B*, disruption of the second motif by substitution of the SUMO acceptor Lys-520 by arginine led to a 50% increase in activity relative to WT AR. In this context, addition of the K386R substitution, which disrupts both motifs, led to a 3.5-fold enhancement. In this context, the clinically isolated P390S and P390D mutations led to a nearly 2-fold enhancement of activity. In contrast, addition of the conservative P390G substitution did not enhance AR activity beyond that observed in the K520R single mutant. As expected, these substitutions had no effect at a promoter bearing a single ARE. Importantly, the functional differences observed in all cases were not due to alterations in the expression of the receptor because Western blot analysis revealed no difference among the different AR forms (Fig. 2, *insets*). Taken together, the above data indicate that the clinically isolated Pro-390 mutations lead to a partial loss of function in the first SC motif of AR.

Because the function of SC motifs is dependent on their SUMO modification, a logical prediction is that the partial loss of SC motif function caused by the clinically isolated mutations is due to alterations in AR SUMOylation. To examine this directly, we used an established cell culture SUMOylation assay (33). As can be seen in Fig. 3*A*, co-expression of HA-tagged SUMO3 led to the appearance of slower migrating AR immunoreactive species in the extracts. Analysis of AR immunoprecipitates also revealed slower migrating AR immunoreactive species (indicated by the *arrowheads*), and these species are also HA-immunoreactive (Fig. 3*A*), confirming their identity as SUMO-modified forms of AR. Disruption of the first SC motif by mutation of the SUMO acceptor Lys-386 to arginine led to a 50% reduction in AR SUMOylation. Notably, the P390S and P390D mutations also reduced AR SUMOylation. Consistent with the partial loss of SC motif function, the effect of these mutants was less severe because SUMOylation was reduced by ~30%. We also examined the effect of the Pro-390 mutations in the context of a disabled second SC motif. As can be seen in Fig. 3*B*, the K520R mutation was less severe than the analogous mutation in the first motif (K386R). Thus, the extent of SUMOylation of the K520R mutant was reduced by 20% compared with WT AR. This is consistent with previous observations suggesting preferential SUMOylation of the first SC motif in AR (17, 33). For the double SC motif mutant form of AR lacking both SUMO acceptor lysines (K386R/K520R), AR SUMO conjugates were severely reduced with the residual modification likely due to a minor SUMOylation site in the C-terminal region of AR (17). Interestingly, addition of the clinically identified P390S or P390D substitutions to K520R led to a substantial loss of AR SUMOylation (~50%). In contrast, addition of the functionally conservative P390G substitution did not

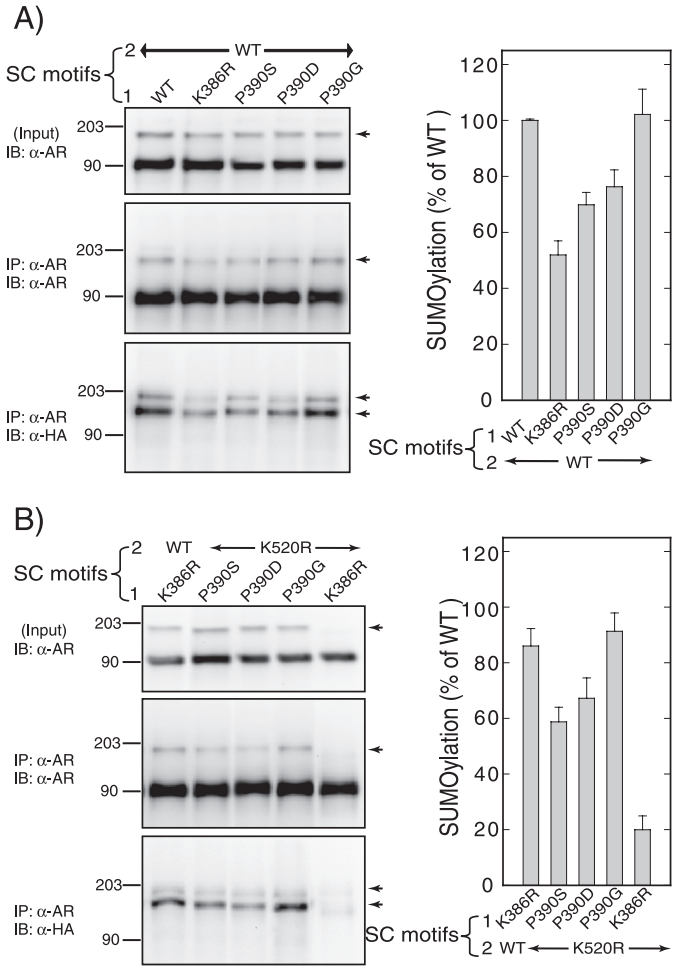

FIGURE 3. **Effect of AIS SC motif mutations on AR SUMOylation *in vivo*.** 293T cells were co-transfected, as described under "Experimental Procedures," with expression vectors for WT AR or the indicated AR SC motif mutants together with the HA-SUMO3 expression plasmid pcDNA3HA SUMO3. Cells were treated 24 h after transfection with 10 nM R1881 or vehicle and processed as described under "Experimental Procedures." *A,* Western blot analysis of cell extracts as well as AR immunoprecipitates (*IP*) from cells expressing the indicated mutants. Blots were probed with AR- or HA (SUMO)-specific antibodies as indicated. *Arrowheads* indicate the positions of singly and multiply SUMO-modified forms of AR, respectively. The stronger HA signal (relative to the anti-AR signal) in the multiply modified upper species is reflective of their higher SUMO to AR stoichiometry. Quantitative analysis of AR SUMOylation (carried out as described under "Experimental Procedures" is shown in the *right panel*. *B,* effect of the same mutations as in *A* but in the context of a disabled second SC motif due to mutation of the SUMO acceptor lysine (K520R) is shown. The data represent the average ± S.E. of at least four independent experiments performed in triplicate and are expressed as a percentage of the SUMOylation observed for WT AR. The average stoichiometry of modification for WT AR was 14.7 ± 3.4%. *IB*, immunoblot.

reduce SUMOylation beyond that observed for K520R alone. Taken together, these results indicate that the clinically isolated Pro-390 mutations interfere with the SUMOylation of the first SC motif in AR and that this defect correlates with the partial loss of function of the first SC motif. The observations clearly confirm the importance of the downstream proline residue for the function and SUMOylation of the SC motif and the ability of glycine to functionally substitute at this position.

A Prostate Cancer Mutation Leads to Loss of SC Motif Function and SUMOylation—A number of mutations in the N-terminal region of AR have been identified in prostate cancer tumor samples (10, 11). Notably, a single glycine 524 to aspartic

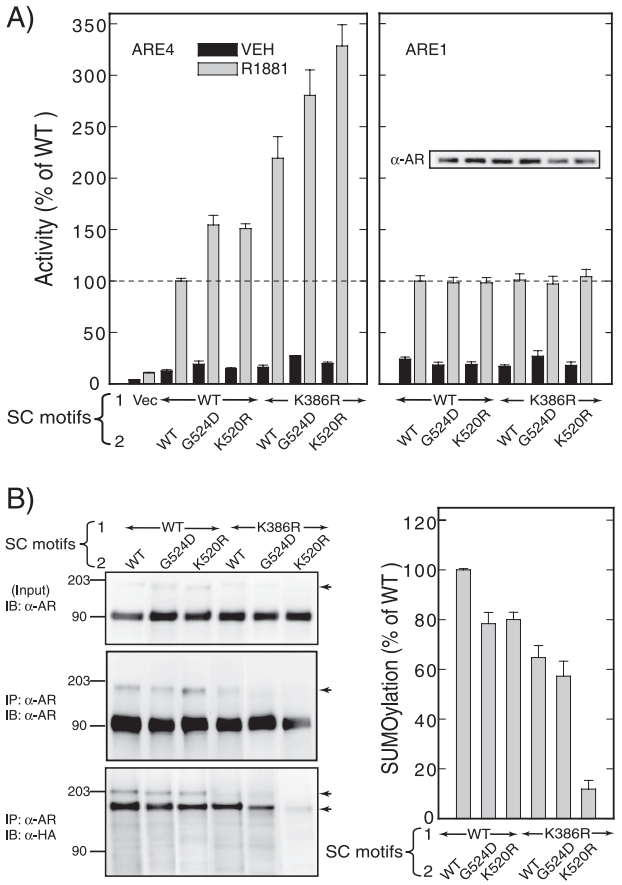

FIGURE 4. **Prostate cancer G524D mutation in the second SC motif negatively affects synergy control and SUMOylation.** *A,* transcriptional activity of AR bearing the indicated mutations in the second SC motif alone or in the context of a disabled first SC motif (K386R) at the $(TAT)_4$-Luc (ARE_4, *left panel*) or $p\Delta(TAT)_1$-Luc (ARE_1, *right panel*) promoters. Cells were co-transfected, treated with 10 nM R1881 or vehicle, and assayed as described under "Experimental Procedures." The data represent the average ± S.E. of four to five independent experiments performed in triplicate and are expressed as a percentage of the corresponding WT AR activity. Western blot analysis of the expression of WT and mutant AR forms is shown in the *inset*. *B,* SUMO modification of the same mutants as in *A*. Analysis and quantitation were as described in Fig. 3. The data represent the means ± S.E. of at least four independent experiments performed in triplicate and are expressed as a percentage of the SUMOylation observed for WT AR. *IB*, immunoblot.

acid substitution was identified in tumor samples of a patient with advanced castration-resistant prostate cancer (10). Gly-524 is positioned immediately downstream of the core of the second SC motif in AR (Fig. 1*B*). We therefore examined the functional consequences of this mutation by determining AR activity at promoter contexts sensitive to synergy control. At the ARE_4 promoter, the activity of AR bearing the G524D substitution was 50% higher than WT AR, an effect indistinguishable from that of the K520R mutation that prevents SUMOylation of the second motif (Fig. 4*A, left panel*). As shown before, disruption of the first motif by the K386R substitution causes a 2-fold enhancement of activity relative to WT AR. In this context, addition of the G524D mutation enhances activity nearly 3-fold relative to WT AR. This effect approaches that of the receptor lacking both SC motif SUMOylation sites (K386R/K520R). Consistent with an alteration of SC motif function, these mutations had no effect at a promoter bearing a single AR-binding site (Fig. 4*A, right panel*). Thus, the prostate cancer-isolated G524D mutation causes a significant loss of the function of the second SC motif in AR. Consistent with the link between SC motif function and SUMOylation, analysis of the effect of the G524D mutation on AR SUMOylation (Fig. 4*B*) revealed a reduction comparable with what was observed when the lysine acceptor site was mutated to arginine (K520R). In the context of a disabled first SC motif (K386R), addition of the G524D mutation further reduced SUMOylation. Consistent with a partial loss of SC motif function, the modification level was above that observed when both SC motif SUMO acceptor lysines were mutated to arginine (K386/K520R). Taken together, these results demonstrate that the proline/glycine residues flanking the core SC motifs of AR play important roles in SC motif function and SUMOylation. This clearly justifies their inclusion in the definition of the SC motif.

SC Motif Mutants Selectively Affect AR Activity at Natural Promoters Harboring Multiple AREs—To examine the functional consequences of synthetic and clinically isolated SC motif mutants at natural regulatory regions, we have taken advantage of recent mapping of AR occupancy across the entire genome (35). Sequence analysis indicates that the majority of AR binding regions harbor one or more instances of a 15-bp motif characteristic of AR binding. Furthermore, 500-bp regions centered around AR occupancy sites serve as functional androgen-response elements in a reporter context (35). We used a panel of 20 such reporters to probe the consequences of disrupting AR SUMOylation at natural regulatory regions occupied by AR in a native context. Our previous characterization of the effects of SC motifs using minimal response elements indicate that SUMOylation-mediated inhibition is preferentially observed under conditions that favor stable DNA binding of the transcription factor (13). Thus, inhibition depends on the presence of multiple high affinity binding sites and is more dramatic when they are closely spaced at the appropriate distances to favor stable binding. In the case of AR and GR, this spacing follows a 10-bp periodicity and is optimal when the receptor dimers bind on the same face of B DNA. Of the 20 natural regions examined, half harbor more than one high scoring 15-bp AR-binding motif, and for nine of these, the SUMOylation-deficient AR mutant (K386R/K520R), which lacks functional SC motifs, displayed significantly enhanced activity relative to WT AR (Fig. 5*A*). In the case of region 6.14, corresponding to the well characterized intronic enhancer of the *FKBP5* gene (37), loss of SUMOylation led to a nearly 3-fold enhancement of activity. Interestingly, this region harbors two high quality AR-binding sequences with one of them being a perfect palindromic match to the consensus sequence. Furthermore, the two sequences are spaced at an optimal 31 bp (center to center) for SC motif function (15). To probe whether the sensitivity to AR SUMOylation depends on functional cooperation between multiple AR-binding sites, we examined the effect of mutating one or both AR-binding sequences in the 7.08 region derived from the *IGFBP1* gene. Consistent with our predictions, disruption of one of the sites led to a large loss of activity, and the remaining activity was insensitive to AR SUMOylation. Disruption of both motifs essentially eliminated AR responsiveness (Fig. 5*A, right panel*). In contrast to the regions harboring multiple AR-binding sequences, analysis of

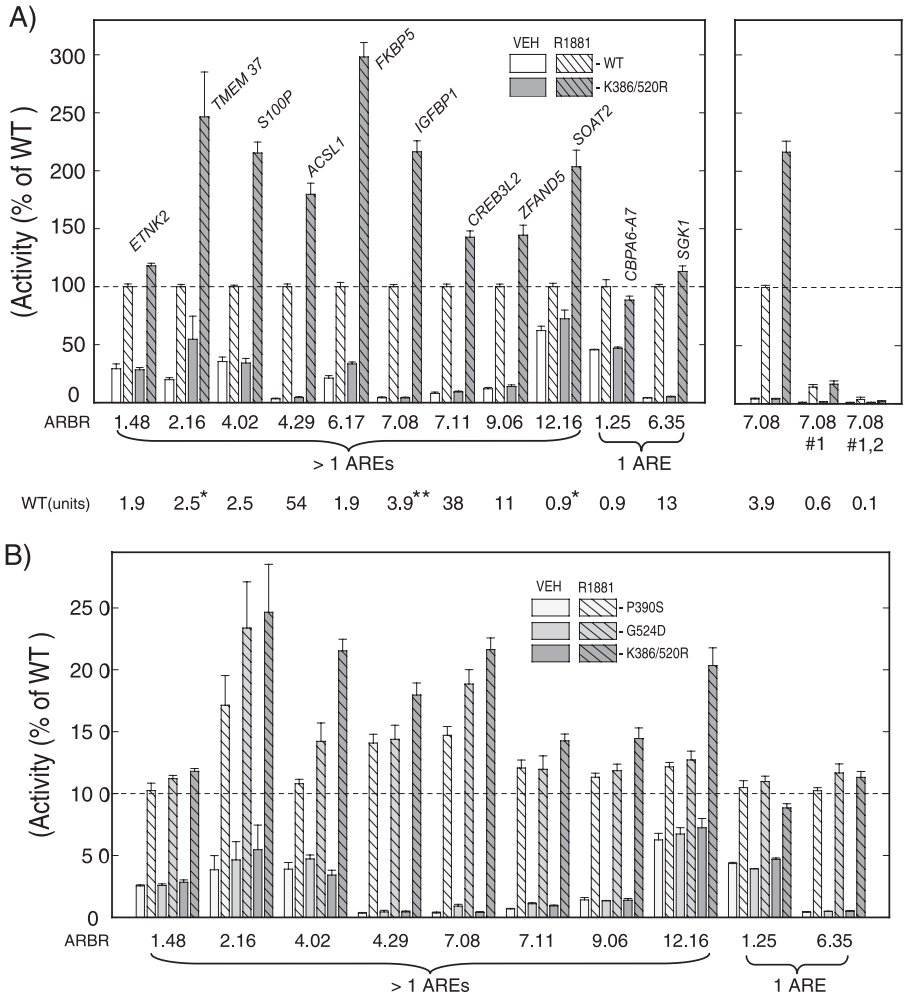

FIGURE 5. **Activity of AR SC motif mutants at natural regulatory regions.** 293T cells were transfected in 96-well plate with a range from 0.01 to 0.1 ng of AR expression plasmids, and 3–30 ng of the indicated reporter as described under "Experimental Procedures." *A,* functional activity of AR bearing WT or mutant (K386R/K520R) SC motif at promoters driven by 500-bp regions centered around the peak *in vivo* occupancy positions. The *1st digit* indicates the chromosome of origin, and the nearest gene is indicated above the *bars*. Regions are grouped by the number of identifiable AR-binding sites. The transcriptional activity of disrupting one or both of the AR-binding sites in region 7.08 is shown in the *right inset*. Single a*sterisk* indicates the transfection of 0.01 ng of the AR expression plasmids and 30 ng of the indicated reporter plasmid. Double *asterisks* indicate the transfection 0.1 ng of the AR expression plasmid and 3 ng of the indicated reporter. All other cells received 0.1 ng of AR expression plasmid and 30 ng of the indicated promoter. *B,* effect of AIS or PC mutations on AR activity at regions bearing multiple or single AREs. The data represent the average ± S.E. of at least four to five independent experiments performed in triplicate and are expressed as a percentage of WT AR activity at each promoter.

those harboring only a single AR-binding sequence revealed that although responsive to androgens the activity was not affected by AR SUMOylation. Two examples are shown in Fig. 5*A*. These results support our previous analysis using isolated AR-binding sequences (15, 33) and suggest that SUMOylation inhibits AR activity emanating from a significant fraction of naturally occurring regions occupied by AR. Having identified natural SUMOylation-sensitive AR binding regions, we examined the impact of the clinically isolated SC motif mutations at these regions. As can be seen in Fig. 5*B*, the AIS (P390S) and prostate cancer (G524D) mutations led to a significant enhancement of activity in nearly all regions. Interestingly, the G524D had a more severe effect, which, for some AR binding regions such as 2.16 and 7.08, approached that of the SUMOylation-deficient double mutant (K386R/K520R). Again, these mutations had no measurable effect at regions harboring a single AR-binding sequence (Fig. 5*B, right*). Taken together, the results indicate that the clinically isolated mutations have significant yet distinct functional consequences for AR activity and that these effects are observed at genomic regions harboring multiple AR-binding sequences.

SC Motif Mutations Enhance AR Induction of Endogenous Genes—The above data indicate that mutations that perturb SUMOylation enhance AR transactivation emanating from a subset of DNA elements occupied by AR. To determine whether these effects translate into enhanced transcription of the genes associated with such elements, we generated isogenic cell lines stably expressing WT or the clinically isolated SC motif mutants as well as the K386R/K520R double mutant and probed AR regulation of the *TMEM37, S100P*, *ACSL1*, *FKBP5,* and *IGFBP1* genes. Notably, the SC motif-deficient K386R/K520R mutant displayed a significantly increased agonist stimulated activity at all five genes, ranging from a 40% increase (*IGFBP1*) to nearly 7-fold in the case of *S100P* (Fig. 6*A*). Notably, the AIS P390S and prostate cancer G524D mutants also showed significantly enhanced activity in nearly all circum-

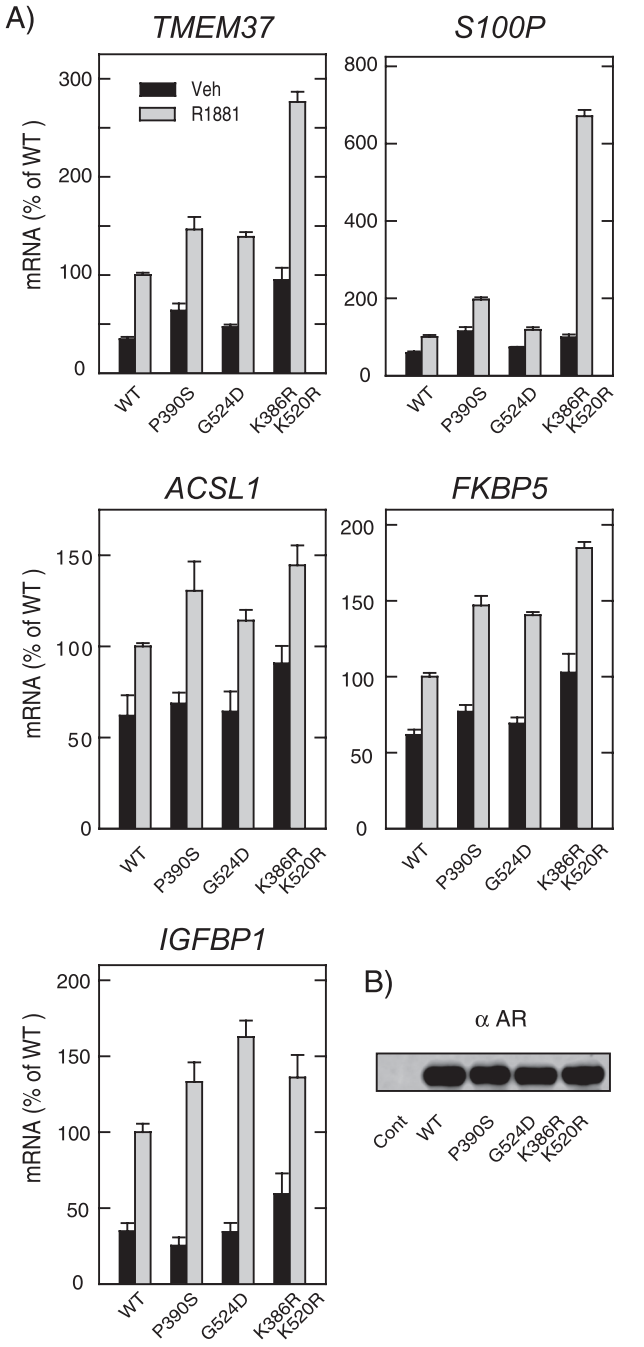

FIGURE 6. **Activity of AR SC motif mutants at endogenous genes.** *A,* cells (HEK293T) stably expressing WT AR or the indicated SC motif mutants were treated with vehicle or the agonist R1881 for 6 h. Data represent the means ± S.E. of three independent experiments performed in duplicate. The average ± S.E. levels of each transcript relative to the *RPL19* gene for the WT receptor in the presence of agonist were as follows: 7.7 ± 2.9 ($\times 10^{-4}$) for *TMEM37*; 11 ± 0.7 ($\times 10^{-4}$) for *S100P*; 32 ± 0.7 ($\times 10^{-4}$) for *ACSL1*; 820 ± 240 ($\times 10^{-4}$) for *FKBP5,* and 0.12 ± 0.05 ($\times 10^{-4}$) for *IGFBP1*. *B,* Western blot analysis of the expression of WT and mutant AR forms.

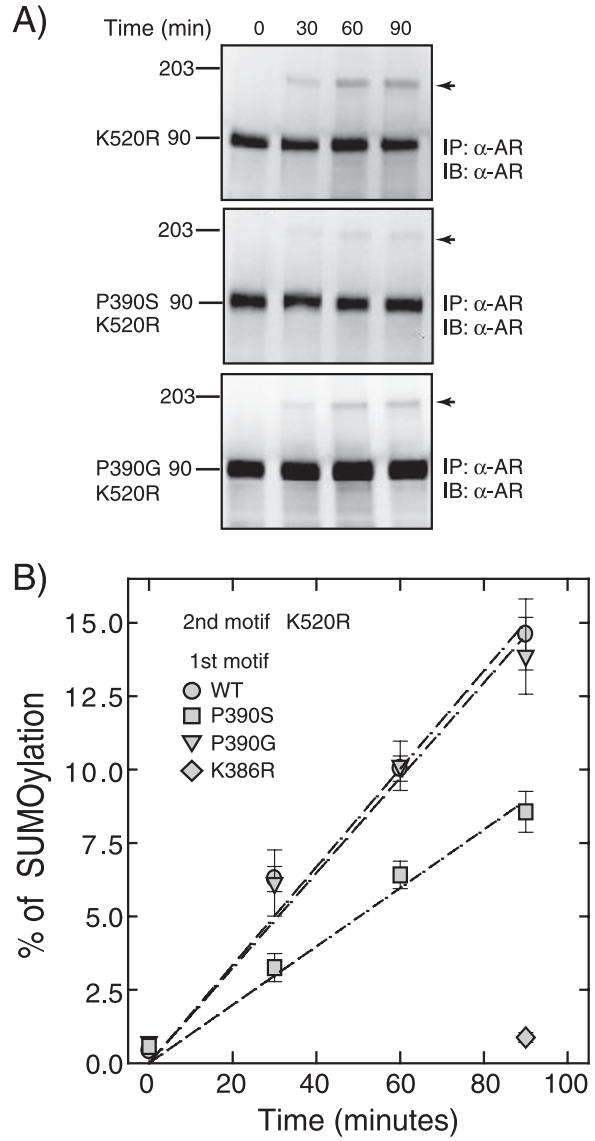

FIGURE 7. **Effect of AIS mutations on *in vitro* conjugation.** *A,* 293T cells were transiently transfected with 5 μg of AR constructs bearing the indicated substitutions and 5 μg of pBSKS(−). Cell lysates were immunoprecipitated (*IP*) with AR antibody and subjected to *in vitro* SUMOylation as described under "Experimental Procedures." Samples were analyzed as in Fig. 3. *Arrowheads* indicate the positions of SUMO-modified forms of AR. *B,* quantitation of conjugation time course. Data represent the means ± S.E. of at least four independent experiments performed in triplicate. *IB*, immunoblot.

stances, except for the *ACSL1* (P390S) and *S100P* (G524D) cases. Furthermore, all receptor forms were expressed at indistinguishable levels (Fig. 6*B*) indicating that the mutations have no detectable effect on AR expression. Taken together, the data clearly indicate that SUMOylation exerts a substantial inhibitory effect on AR activity at multiple endogenous genes and that partial loss of this modification in the clinically isolated mutants has a significant impact on AR induction of susceptible genes.

Decreased SUMOylation in AIS Mutations Is a Selective Defect in Conjugation—Because SUMOylation is a reversible process, the lower steady-state levels of modification observed for the clinically isolated mutants could be due to reductions in the rate of conjugation, an enhanced rate of deconjugation, or a combination of both effects. To investigate the mechanistic role of the flanking Gly/Pro residues in the forward SUMO conjugation reaction, we used a reconstituted *in vitro* SUMOylation system using purified recombinant components and immunopurified AR forms as substrates. We focused on the first SC motif because this is the primary SUMOylation site and examined the time course of SUMO conjugation of an AR form lack-

ing the second modification site (K520R). As can be seen in Fig. 7*A*, under these conditions, the time-dependent accumulation of a single slowly migrating modified species is readily detected. Notably, quantitative analysis revealed that the P390S AIS mutation reduced the rate of conjugation to nearly 50% compared with a WT first motif (Fig. 7*B*). In contrast, modification of a mutant bearing the conservative P390G substitution progressed at a rate indistinguishable from that of an intact first motif. The signal detected was derived from modification of the first motif because mutation of the acceptor lysine in this context abolished modification (K386R/K520R). Thus, the above results indicate that the P390S mutation renders the first motif a poorer substrate for conjugation and that glycine can substitute for proline at this position, presumably due to their comparable propensity to terminate secondary structure elements. To examine whether the P390S AIS mutation alters SUMO deconjugation, we established a quantitative *in vitro* deconjugation assay using the purified catalytic domain of the SUMO-specific protease SENP2 and immunopurified SUMOylated AR forms as substrates. Again, we focused on the first motif by examining AR species lacking the second SUMOylation site (K520R). As can be seen in Fig. 8*A*, when AR immunoprecipitates were treated with WT SENP2 at 23 °C, a time-dependent loss of the conjugates was readily detected, whereas a catalytically inactive (C68S) form of SENP2 failed to do so. Quantitative analysis of the data (Fig. 8*B*) indicated that deconjugation was affected neither by the P390S AIS mutation nor by the conservative P390G substitution. Taken together, the above results indicate that the effect of AIS mutation (P390S) on SC motif function and SUMOylation was not due to enhanced deconjugation but to a selective impairment in the forward SUMO conjugation reaction.

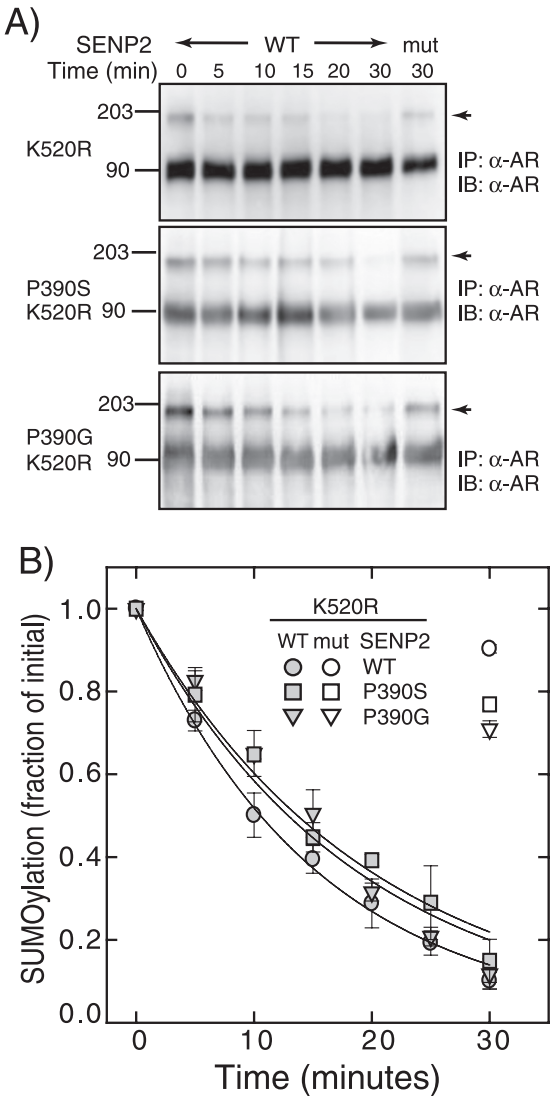

FIGURE 8. **Effect of AIS mutations on *in vitro* deconjugation.** *A,* 293T cells were transiently transfected with 5 μg of AR constructs bearing the indicated substitutions and 5 μg of HA-SUMO-3 expression vector. AR immunoprecipitates were treated with WT or mutant (C548S) SENP2 as described under "Experimental Procedures," and samples were analyzed as in Fig. 3. *Arrowheads* indicate the positions of SUMO-modified forms of AR. *B,* quantitation of deconjugation time course. Data represent the means ± S.E. of at least four independent experiments performed in triplicate. *IB*, immunoblot.

DISCUSSION

Defective SUMOylation as the Cause of AR-based Diseases—The functional role of SUMOylation for multiple transcription factors has been well established *in vitro*, but the impact of this modification in their *in vivo* function is less clear. The present analysis of AR mutations associated with androgen insensitivity, testicular cancer, and prostate cancer clearly indicates that AR SUMOylation is an important regulatory mechanism that has a major impact in the physiological role of this receptor. An important observation is that AR activity at a significant fraction of natural AR binding regions is affected by mutations that prevent or partially affect SUMOylation of AR. Notably, these regulatory effects have a direct consequence on the AR regulation of the genes associated with such regions. It is important to note that in many cases multiple AR binding regions are found in the vicinity of a given AR-regulated gene and only a subset are likely to be AR SUMOylation-sensitive. For the cases we have examined, because the overall gene response is affected by AR SUMOylation, this suggests that the SUMOylation-sensitive regions are the major contributors to the overall AR response sensitivity of these genes. This is consistent with our previous studies (15, 33) that indicate that SUMO-mediated inhibition is most dramatic at genomic regions harboring multiple, closely spaced AR-binding sites, which are also particularly effective at supporting AR-mediated activation. The data on these endogenous genes therefore supports our view that stable assembly at the promoter makes transcription factors particularly susceptible to SUMO-mediated inhibition (13). As a corollary, the correspondence between isolated regions and endogenous genes also indicate that the mechanism(s) involved are unlikely to depend on a chromosomal context *per se* or on direct sequence proximity to the transcription start site. These properties indicate that the mechanism(s) by which SUMOylation limit AR activity are likely to be independent of chromatin features or processes that are exclusive to the chromosomal environment. Given that the extent of SUMOylation is likely to be different in specific cell types or pathophysiological conditions, coupled with the sequence context dependence of the effects, argues that the clinically isolated mutations are likely to alter major AR-regulated genes with a unique spec-

trum and magnitude depending on the particular cellular and physiological context.

It is also notable that mutations associated with androgen insensitivity syndrome and testicular cancer map to the first SC/SUMOylation motif, whereas the prostate cancer mutation targets the second motif. One possibility is that disruption of the first motif affects preferentially the expression of genes relevant to the AIS phenotype, whereas those affecting prostate cancer may be more sensitive to the second motif. In this regard, the analysis of natural AR binding regions indicates that the first motif seems to have a more significant effect. On average, disruption of the first motif (K386R) led to an enhancement reaching 53% of that observed for the double mutant K386R/K520R, whereas the value for disruption of the second motif alone was 25%. This difference however is not as evident for the endogenous genes. A clear point is that although the overall stoichiometry of SUMOylation is low, even relatively subtle changes in SUMOylation can cause a significantly altered pattern of AR activity.

AR SUMOylation and Androgen Insensitivity—AIS is generally associated with a loss of androgen signaling function. It is therefore counterintuitive that the P390S mutation, which leads to enhanced AR activity, is associated with a clinical phenotype of androgen insensitivity. The fact that the P390S mutation has been repeatedly and independently identified in unrelated individuals with AIS (3, 28–30) and that a P390D mutation is also associated with AIS (32) argue strongly that the mutations are indeed responsible for the phenotype. It is important to note that the germ line nature of the mutation means that the carriers have been exposed to altered AR activity throughout development, including the critical early prenatal periods when the function of the hypothalamic-pituitary-gonadal axis is established in an AR-dependent manner (38). In this regard, recent data indicate that prenatal exposure to elevated androgens in ovines leads to subsequent defects in testicular development and function reminiscent of androgen insensitivity, including lower testicular mass as well as decreased sperm count and motility (39, 40). Although the mechanism of these alterations is not fully understood, it is possible that androgen excess could lead to early reprogramming of the hypothalamic pituitary gonadal axis, which is an AR-dependent process (38). In this regard, analysis of hormonal levels in P390S patients (3) indicated elevated gonadotropins but near normal testosterone levels. This suggests potential defects in Leydig cell function or responsiveness to gonadotropins or alterations in feedback inhibition. Examining the effects of AR SUMOylation on Inhibin synthesis or direct feedback processes in the brain may be informative. Because the effects of the mutation are promoter context-dependent and AR SUMOylation stoichiometry may be tissue-selective, it is also possible that an altered AR activity pattern in the testis can lead to a similar outcome as a generalized reduction in AR activity. Further characterization of the functional deficit in these patients may shed light in this regard. It is also notable that the isolation of the P390S mutation in a patient with testicular cancer (31) is consistent with the observation that AIS is a clear risk factor for this malignancy (41, 42). Although the exact basis for this association is unclear, elevated luteinizing hormone levels, as observed in AIS, have been implicated in testicular cancer (43). Taken together, the data clearly support the view that changes in AR SUMOylation can have significant pathophysiological consequences.

AR SUMOylation in Prostate Cancer—The enhanced activity phenotype of the G524D mutant is consistent with the central role of AR in providing the major survival and proliferative drive that sustains the progression of prostate cancer (5). Because SUMOylation inhibits both the ligand-activated as well as the basal ligand-independent activity of AR,[4] the G524D mutation is consistent with a selection mechanism to maintain AR activity even in the absence of androgens. This is in keeping with the mutation being isolated from a patient that relapsed after castration (10). The role of the second SC/SUMOylation motif in prostate cancer is also supported by recent sequencing of rapid autopsy samples where mutations in the vicinity of the motif were isolated multiple times in different tumor samples (12). Whether these mutations alter SUMOylation in a manner similar to the G524D mutation will require further characterization.

The inhibitory effect of SUMOylation on AR and other transcription factors argues that mechanisms that keep SUMOylation low may provide a selective advantage for prostate cancer progression. This view is also supported by gene expression studies that have shown a significant overexpression of the SUMO protease SENP1 in advanced stages of prostate cancer (44, 45). Mechanistically, AR is a direct activator of the *SENP1* gene (45), and SENP1 is a positive regulator of AR activity (46, 47), in part by serving as the principal SUMO protease responsible for cleaving SUMO from AR (47). Thus, loss of AR SUMOylation would lead to enhanced SENP1 induction, which in turn would further limit AR SUMOylation. Such a feed forward mechanism can therefore suppress overall SUMOylation and maintain AR activity. Notably, recent data argue that enhanced SUMOylation is a strong signal for induction of senescence-mediated growth arrest (48, 49). Therefore, suppression of SUMOylation may provide additional selective advantages for prostate cancer progression beyond supporting an aberrant AR transcriptional program.

Role of Flanking Residues in SC/SUMOylation Motifs—A notable finding from these results is that the clinically isolated mutations affect the flanking Pro/Gly residues but not the SUMO acceptor Lys residues themselves. Although this may be due to sampling bias, it is consistent with the intrinsically higher rate of mutations expected for Pro (5-fold) and Gly (4-fold) relative to Lys residues (50). It could also indicate that a partial loss of SUMOylation is more tolerable. Examination of the mutation patterns of larger cohorts will be informative in this regard. In our initial functional definition of SC/SUMOylation motifs (15), we noted the presence of Pro (and subsequently Gly) residues in the vicinity of the core SC motif sequence. Given the propensity of these residues to terminate secondary structure elements, we interpreted their presence to reflect the location of SC motifs in exposed loops. The selective effect of the clinical mutations to interfere with the conjugation

[4] J. A. Iñiguez-Lluhí, unpublished observations.

reaction coupled with the interchangeable nature of Pro and Gly residues provides experimental evidence for this idea and is supported by the crystal structures of known SUMOylated proteins where the flanking Pro and Gly residues terminate α-helices and delineate the exposed SC/SUMOylation motif (51–53). The data clearly validate the inclusion of these flanking residues in the definition of SC motifs. Because the core SUMOylation motif is only four residues long and has relatively low information content, commonly used search algorithms based solely on the core tend to have a high false-positive rate. We have found that inclusion of the nearby Pro/Gly feature significantly improves the detection of *bona fide* SC/SUMOylation motifs.[5] Interestingly, the clinical relevance of mutations in Pro/Gly residues flanking SUMOylation motifs may extend to other proteins because a polymorphism substituting a leucine for the downstream proline of the second SUMOylation motif of the voltage-gated ion channel Kv1.5 (54) is present in 1.1% of African-Americans and is responsible for clinically significant resistance to antiarrhythmic drugs (55).

Novel SUMO-based Therapeutic Opportunities—Together with the demonstration that AR SUMOylation reduces aggregation in Kennedy disease (33), the current data support the view that alterations in AR SUMOylation play a significant role in the principal AR-based diseases and raise the possibility that therapeutic interventions to modify this pathway may be effective in their treatment. Based on our data, strategies that enhance AR SUMOylation would be desirable particularly for prostate cancer and Kennedy disease. Inhibition of SUMO proteases may be a suitable approach, especially because the P390S mutation does not interfere with deSUMOylation. In this regard, the up-regulation of SENP1 in prostate cancer makes it an attractive candidate for drug design. Clearly, the success of ubiquitin-proteasome-based cancer therapies (56) offers a clear precedent and illustrates how a widespread and essential biochemical process can also offer specific therapeutic opportunities.

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